

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/10 // C07K 14/47		A1	(11) International Publication Number: WO 99/58646 (43) International Publication Date: 18 November 1999 (18.11.99)
(21) International Application Number: PCT/EP99/03075 (22) International Filing Date: 5 May 1999 (05.05.99) (30) Priority Data: MI98A001004 8 May 1998 (08.05.98) IT		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(71) Applicant (<i>for all designated States except US</i>): GENERAL S.P.A. [IT/IT]; Via Olgettina, 58, I-20132 Milano (IT). (72) Inventor; and (75) Inventor/Applicant (<i>for US only</i>): MAVILIO, Fulvio [IT/IT]; Via Olgettina, 58, I-20132 Milano (IT). (74) Agent: MINOJA, Fabrizio; Bianchetti Bracco Minoja S.r.l., Via Rossini, 8, I-20122 Milano (IT).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: GENETICALLY-MODIFIED FIBROBLASTS AND THE USE THEREOF (57) Abstract <p>A method for the preparation of genetically-modified fibroblasts expressing a muscle lineage commitment gene, and the use thereof for the treatment of genetic defects or for the expression of therapeutic proteins.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

GENETICALLY-MODIFIED FIBROBLASTS AND THE USE
THEREOF

The present invention relates to an ex-vivo method for the myogenic conversion of genetically-modified fibroblasts, for use in the correction of congenital defects of the muscular system, such as primary monogenic myopathies, or in the production of therapeutical proteins.

5 Furthermore, the invention relates to the use of genetically-modified fibroblasts for the preparation of compositions for cell implants for the treatment of muscle pathologies with a genetic aetiology, or for the in vivo secretion of recombinant proteins of therapeutical importance. In particular, the invention is valuable for the treatment of muscular

10 dystrophies, a heterogeneous group of severe degenerative diseases of the muscle caused by mutations in the genes encoding for the membrane-associated protein dystrophin or for other members of the dystrophin-associated protein complex connecting the muscle fiber cytoskeleton with the extracellular matrix.

15 Until now, the attempts to correct muscular dystrophy with gene therapy based on the implantation of muscles or other tissues stem cells, have been thwarted by enormous difficulties in the isolation of a suitable number of myogenic cells to be genetically-modified or to the poor yield of phenotypic conversion. By way of example, the difficulty in obtaining

20 a reasonable number of satellite cells to be genetically-modified is a limit to gene therapy of patients with Duchenne muscular dystrophy or with Becker muscular dystrophy (Salvatori, G., et al., 1993; Webster, C. et al., 1990). Further problems related to the implantation of genetically-modified cells in dystrophic patients are the immunogenicity of viral

vectors, the difficulty in developing an effective administration route and the poor survival of injected cells. Furthermore, the expression of dystrophin itself has been reported to induce cellular and/or humoral immune responses in dystrophin-lacking patients (New England J. of Medicine 333, 732-733, 1995).

It has been known for some time that forced expression of MyoD, and related members of the myogenic family of basic helix-loop-helix transcription factors, would activate myogenesis in non-muscle cells (Davis, R. L., et al., 1987; Cossu, G., et al., 1996; Cossu, G., 1997). The 10 Applicant itself has recently reported that fibroblasts undergo myogenic differentiation when co-cultured with myogenic lines (C2C12) or primary cells, but not with other cell types (Breton, M. et al., 1995; Gibson, A.J., et al., 1995; Salvatori, G. et al., 1995). WO 95/12979 discloses a general method for inducing cell differentiation to a novel phenotype, for example 15 muscular phenotype, by inserting the genic sequences of factors such as MyoD, myogenin, Myf-5 and MRF4 into the cells. The same document neither considers any possibility to convert genetically-modified cells to the myogenic phenotype in order to make them suitable for gene therapy, nor envisages the possibility to carry out an "ex vivo" myogenic 20 conversion.

GB 2293604 discloses the use of fibroblasts in the treatment of muscular pathologies, wherein fibroblasts themselves can be transformed with a therapeutically active gene, for example the dystrophin gene, whereas the possibility to induce differentiation by means of myogenic 25 factors has not been considered.

In light of what stated above, it appears remarkably advantageous to rely on a method which allows a high number of cells to be converted

to the myogenic phenotype, after being genetically-modified ex-vivo, in order to correct a genetic defect, such as muscular dystrophy, or any other dysfunction related, for instance, to insufficient or incorrect production of a protein or a plasma factor or other secreted or circulating proteins, as in 5 the case of diabetes, hemophilia, pituitary dwarfism.

It has now been found that it is possible to convert genetically-modified fibroblasts to the muscular phenotype, with a high-efficiency process, so as to effectively produce therapeutical proteins or proteins able to correct the genetic defects, particularly muscular dystrophy.

10 According to a first aspect, the invention provides a high-efficiency method for the preparation of genetically-modified fibroblasts expressing muscle lineage commitment genes, which comprises:

- a) ex-vivo transduction of fibroblasts with a therapeutic gene or a gene capable of correcting a genetic defect;
- 15 b) transient expression of the muscle lineage commitment gene in fibroblasts transduced as at point (a), through transformation of the cells with a high efficiency DNA transfer method, preferably a viral vector, more preferably selected from baculovirus, adeno-associated viruses and, most preferably, adenovirus, or with other 20 high efficiency DNA transfer means, such as liposomes, nucleoprotein complexes, polyethylenimines, wherein the gene of the muscle lineage commitment is under the control of a strong promoter, preferably a viral promoter.

"Muscle lineage commitment gene" means any gene capable of 25 converting fibroblasts to the myogenic phenotype, particularly MyF-5, MRF4, myogenin and, preferably, MyoD genes.

Therapeutical genes which can be transduced into fibroblasts

before the myogenic conversion are, for example, genes involved the various types of muscle dystrophies, such as sarcoglycan, emerin and, preferably, dystrophin genes, or genes coding for circulating plasma proteins, such as coagulation factors or insulin, for hormones, such as 5 growth hormone, or other genes, such as hypoxanthine-guanine phosphoribosyl transferase, adenosine deaminase, purine nucleoside phosphorylase, glucocerebrosidase, low density lipoprotein receptor, phenylalanine hydroxylase, arginine succinate synthetase and aryl-sulfatase.

A second aspect of the invention relates to genetically-modified 10 fibroblasts, transiently expressing a gene of the muscle lineage commitment, preferably MyoD, obtained by the method of the invention.

The conversion of fibroblasts to the myogenic phenotype by transient expression of a muscle lineage commitment gene, proved to be surprisingly more effective than other alternative techniques. In fact, 15 co-culture of fibroblasts with satellite cells, treatment with dexamethasone and transfection with calcium phosphate, lipofectamine, or electroporation of the same plasmid expressing MyoD, induced myogenic conversion in a percentage ranging from 1% to 14%, in comparison with values higher than 70% in case of infection with a recombinant adenoviral 20 vector. Furthermore, the efficiency of conversion was found to increase linearly for m.o.i. ("multiplicity of infection") values from 500 to 2000, whereas cytotoxicity at maximum m.o.i. values, remained within acceptable limits. Similar results were obtained with human or murine 25 fibroblasts, whereas as far as tissutal fibroblasts are concerned, dermal fibroblasts gave better results. One main advantage of the method of the invention is that transient expression of the exogenous muscle lineage-commitment gene activates the corresponding endogenous gene, therefore

irreversibly committing cells to myogenesis, thus rendering constitutive expression of the transgene unnecessary. The actual myogenic conversion of fibroblasts transiently expressing exogenous MyoD has been confirmed by immuno-histochemistry, electron microscopy and gene expression analysis of different muscle-specific markers, such as myosin light and heavy chains, acetylcholine receptor α -subunit, M creatine kinase, myogenin and MyoD.

The high efficiency of the method allows the preparation of genetically manipulated fibroblasts in which a therapeutic gene, or a gene correcting the genetic defect in the muscle has been inserted, which may be stably converted to the myogenic phenotype. In fact, conversion to the muscle phenotype prevents cell replication and provides stable implants, based on the use of muscle fibers.

Furthermore, the efficiency of the method as well as the stability of the resulting product allow to successfully carry out gene-therapy, in which genetically-modified fibroblasts expressing a gene of the muscle lineage commitment, after appropriate ex-vivo treatment, are injected into muscle tissue where they are capable of regenerating the muscle fibers and express the correct gene.

A number of evidences proved that fibroblasts treated ex-vivo as herein disclosed are able to regenerate muscle fibers in mice, indistinguishable from those originating from primary myogenic (satellite) cells, with a long-lasting effect. Moreover, after treatment, only antibody mediated immune response against proteins of adenoviral infected fibroblasts has been detected. No cell-mediated immune response has been observed at the site of injection of the modified fibroblasts. This is a significant advantage in gene therapy, as one of the major problems

associated to the in vivo use of adenoviral vectors is the induction of significant, T-cell-mediated immune responses against both the viral and transgenic proteins.

According to a further aspect, the invention relates to the use of 5 fibroblasts obtained by the above described method, for "ex vivo" gene therapy or for the preparation of compositions for stable cell implants based on muscle fibers.

A typical application of the ex-vivo gene therapy comprises, for example, isolating dermal fibroblasts from a patient suffering from a 10 genetic disease which alters the muscle structure or functionality, such as dystrophy, expanding the cultured cells, transducing the cells ex-vivo with a retroviral vector containing the correct gene, and inducing myogenesis by infection with an adenoviral vector, or with another vector as mentioned above, containing the gene of the muscle lineage 15 commitment, and re-implanting modified cells into the muscle tissue.

Another typical approach for the preparation of cell implants based on muscle fibers comprises isolating dermal fibroblasts from a patient suffering from a disease characterised by, for example, lack of a plasma protein (such as diabetes, pituitary dwarfism, hemophilia), expanding the 20 cultured cells, transducing the cells ex-vivo with a retroviral vector containing the therapeutic gene, and inducing myogenesis by infection with an adenoviral vector, or with another vector as mentioned above, containing the muscle lineage commitment gene, encapsulating the modified cells in the most suitable implantation matrix and implanting the 25 preparation in the preferred body site. In this specific application, induction of myogenesis and subsequent differentiation into muscle tissue is not aimed at the restoration of muscle functionality, but to the stability

of the implant by arrest of cell division.

As further applications of the present invention, its use in zootechnic field can be envisaged, to induce a specific characteristic in animals, for example an increase in weight, by production of a hormone or any other substance by the cells treated ex vivo with the herein disclosed method and then re-implanted into the animal; or the creation or use of animal models of human pathologies for the study of novel therapeutical procedures to be carried out with the method of the invention.

10 The following examples illustrate the invention in greater details.

Example 1

Preparation of genetically-modified fibroblasts expressing MyoD.

Fetal fibroblasts were isolated from skin and skeletal muscle of C3H mouse embryos (15-17 days) or legally aborted human fetuses (8-12 weeks) as described in Salvatori et al., *Human Gene Ther.*, 1993, 4:713-723, or human dermal or muscle fibroblasts were obtained from tissues of patients undergoing post-traumatic surgery. In this case tissues were finely minced with scissors, digested with 2 mg/ml dispase, 0.1 mg/ml collagenase in phosphate buffered saline (PBS) for 45' at 37°C, washed in 20 RPMI medium, and pipetted to obtain a single-cell suspension.

Murine bone marrow fibroblasts transgenic for the lacZ gene with nuclear localization, were obtained by resuspending and plating cells flushed from the long bones of 8-10-wk-old MLC3F/nlacZ mice. Human bone marrow fibroblasts were obtained from healthy donors after removal 25 of non-adherent cells. All cells were grown in RPMI supplemented with 15% Fetal calf serum (FCS), 1% gentamycin and 0.3 mM β -mercaptoethanol (growth medium). Human bone marrow fibroblasts were

supplemented with 2 ng/ml bFGF.

Myogenic differentiation was induced by shifting the cells to RPMI supplemented with 2% horse serum (differentiation medium). Fibroblasts were purified by sub-culture (at least two rounds) in growth medium.

5 Removal of myogenic cells was routinely verified by immunocytochemical staining of a cell aliquot sub-cultured for 5 days in differentiation medium.

For transduction, fibroblasts (from third to tenth passage) were infected with a replication-defective retroviral vector (LBSN) expressing 10 a cytoplasmic β -galactosidase gene under the LTR promoter as described (Salvatori, G., et al., 1993).

For the subsequent step (transient expression of MyoD), cells were treated with the Ad5-derived, E1A-deleted adenoviral vector expressing the full-length murine MyoD c-DNA under the transcriptional control of 15 Rous Sarcoma Virus (RSV) LTR (Murry, C. E., et al., 1996). In some control experiments, cells were transfected by standard precipitation techniques with calcium-phosphate or lipofectamine (Dotap) with 10 μ g of the PMC11 plasmid, containing the MyoD cDNA under the control of the CMV promoter.

20 Alternatively, cells were electroporated with 6 μ g of the same plasmid in growth medium at 120 V, 960 mF. After transduction, cells were grown for 24 hrs in growth medium and then either shifted to differentiation medium for 3-4 days or injected *in vivo*.

In some experiments, cells were pre-labelled with 0.5 mCi/ml of 25 [14C] thymidine (Amersham) for 24 h and then exposed to the adenoviral vector expressing MyoD and survival was measured by counting residual cpm incorporated.

Example 2**Implantation of MyoD-converted fibroblasts into mouse muscles.**

1 or 2 millions of transduced (or transgenic) human or murine fibroblasts, converted by transient expression of MyoD, were trypsinized, resuspended in 20-50 µl of PBS and injected into a single site of the regenerating tibialis anterior muscle of either syngeneic (C3H) or immunodeficient (scid/bg) mice which had received a 30-µl injection of 10.5 M cardiotoxin (Latoxan) 48 hrs earlier.

For some experiments, myogenically converted mouse fibroblasts were labelled with DiI dye 1 hr before injection *in vivo* (a 0.5% solution of DiI in absolute ethanol was diluted just before use in 0.3 M sucrose to a final concentration of 0.05%).

Mice were sacrificed after various periods of time, and muscles were cryo-sectioned and stained for β-galactosidase activity or processed for immuno-histochemistry. At the time of sacrifice, serum was collected from C3H mice and reacted with adeno-infected or control cells at various dilutions, followed by a fluorescein-conjugated anti-mouse IgG secondary antibody.

Example 3

20 **Immunocytochemistry, electron microscopy and RNA analysis techniques.**

Immunofluorescence analysis was carried out as described (Cusella - De Angelis, M.G., et al., 1994) using the following antibodies: MF20, a monoclonal antibody which recognises all sarcomeric myosins (Bader, D., et al., 1982); a rabbit antiserum against sarcomeric proteins (Tajbakhrsh, S., et al., 1994); an anti-MyoD polyclonal antibody (Hasty, P., et al., 1993); BD5, a monoclonal antibody which recognises slow myosin heavy

chains (Yasin, R., et al., 1977); a rabbit polyclonal antibody against human fetal myosin (Edom, F., et al., 1994); an anti-bromo-deoxyuridine (BrdU) monoclonal antibody; anti-leu, anti-CD4 and anti-Mac3 rat anti-mouse leukocyte antigen antibodies. Briefly, cells cultures or cryostat sections were fixed with 4% paraformaldehyde for 10 min at 4°C, washed 3X in PBS, and incubated at 4°C with the primary antibodies in PBS + 1% bovine serum albumin (BSA). BrdU labelled cell-cultures were treated for 10 min at rt with 2M HCl and washed 3X with PBS before incubation with the anti-BrdU antibody.

10 After the first incubation, cells or sections were washed 3X with PBS + 1% BSA and incubated for 1 hr at rt with rhodamine-conjugated goat anti-mouse Ig or with a fluorescein-conjugated goat anti rabbit Ig (1:100 dilution).

15 Cultures or sections were then washed, mounted in 75% glycerol/PBS (pH 7.5), and observed under a Zeiss Axiophot epifluorescence microscope.

For electron microscopy cells were washed in PBS, fixed in 2% glutaraldehyde in 0.1 M Millonig's buffer (pH 7.4) for 1 hr at 4°C, post-fixed for 1 hr in 1% buffered osmium tetroxide and dehydrated in graded 20 alcohol. Cells were treated with propylene oxide, embedded in Epon 812 and cut into ultrathin sections. The ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss 109 electron microscope.

For RNA analysis, RNA was extracted by the method of 25 Chomczynski et al. (Chomczynski, P., et al., 1987), run in 15 µg aliquots on 1% agarose/formaldehyde gels, and transferred by capillary Northern blotting to nylon membranes (Amersham Hybond-N).

Filters were cross-linked for 2 hrs at 80 °C under vacuum, and hybridized to [³²P]-labelled probes for MyoD and myogenin (Bober, E., et al., 1991), MLC1F and MCK (Lyons, G. E., et al., 1991), Ach-receptor α-subunit (25) under standard conditions (26).

5 - Example 4

Analysis of in vitro converted fibroblasts expressing the myogenic phenotype.

In order to compare the adeno-MyoD-induced phenotype with that of primary myogenic cells, a number of muscle-specific markers were 10 analysed by immuno-cytochemistry, electron microscopy and gene expression analysis, in human and murine converted dermal fibroblasts, and in differentiating satellite cells of comparable age.

All differentiated myotubes derived from fusion of converted murine newborn dermal fibroblasts express fast embryonic myosin heavy 15 chains, while a fraction of them also express slow myosin heavy chains, while a fraction of them also express slow myosin heavy chains, as observed in myotubes derived from satellite cells.

Similarly, myotubes derived from human fetal dermal fibroblasts show a well organized initial sarcomerogenesis, with aligned sarcomeres 20 and patterned Z lines, as reported for myotubes derived from primary myogenic cells, which never complete sarcomerogenesis in vitro.

In order to verify whether the introduction of MyoD in primary fibroblasts would also activate transcription of genes responsible for membrane and metabolic muscle-specific functions, the Applicant 25 measured by Northern blotting the expression of RNAs coding muscle-specific proteins such as the acetylcholine (ACh) receptor α-subunit, M creatine kinase (MCK), as well as MyoD, myogenin, and myosin light

chain 1 fast (MLC1F) chosen as positive controls.

No detectable level of any of these messages was detected in unconverted fibroblasts; on the other hand, these mRNAs were expressed at comparable levels in myotubes derived from converted fibroblasts and 5 in myotubes derived from satellite cells of corresponding age.

Thus, under all the parameters analysed *in vitro*, myotubes originating from converted fibroblasts cannot be distinguished from those derived by primary myogenic cells.

Afterwards, the Applicant investigated whether fibroblasts exposed 10 to the MyoD adenoviral vector would still maintain the capacity to divide, since MyoD overexpression has an anti-proliferative effect. Murine and human fetal skin fibroblasts were infected with the MyoD adenoviral vector for 3 hrs at an m.o.i. of 2,000 in serum-free medium, cultured for 12 hrs in growth medium containing 10 mM BrdU, fixed at 12, 24, 48 and 15 72 hrs after infection, and double-stained with anti-MyoD and anti-BrdU 20 antibodies.

24 hrs after exposure to the adenoviral vector, only a few cells expressing MyoD had incorporated BrdU, while no double-positive cells were detected at 48 and 72 hrs. Expression of MyoD therefore blocks cell division in converted fibroblasts.

Example 5

Myogenic conversion efficiency of human and murine fibroblasts of different origins

Fibroblasts were isolated, expanded, infected with the MyoD 25 adenoviral vector for 3 hrs at an m.o.i. of 2,000, cultured for a further 24 hrs and then induced to differentiation as described. Table 1 shows the conversion % values.

The conversion efficiency was measured as percentage of cells which expressed sarcomeric myosin heavy chains, as shown by staining with anti-myosin antibody. (MF20). Values shown are the average of two separate experiments, each performed in triplicate.

Table 1

	Murine		Human		
	Fetal	Adult	Fetal	Adult	
10	Derma	70	56	65	42
	Muscle	43	44	40	32
	Bone marrow	ND	6	ND	6

ND: not done.

15 Example 6

Survival of MyoD-converted fibroblasts and satellite cells (from MLC3F/LacZ mice) after injection into regenerating Tibialis Anterior muscle (TA) of SCID/bg mice.

20 $20 \mu\text{l}$ containing 1×10^6 cells were injected into a single site of regenerating TA of SCID/bg mice.

At the indicated times, mice were sacrificed and successive $15 \mu\text{m}$ cryostat sections were prepared from the TA muscle. 1 every 5 slides was X-gal stained. Nuclei were counter-stained with Hoechst. The X-gal stained sections, were scored for the number of $\beta\text{-gal}^+$ nuclei and the obtained values were multiplied x 5 (total No. of nuclei). In addition $\beta\text{-gal}^+/\text{total nuclei}$ in $200 \mu\text{m}^2$ areas was counted. Results are reported in Table 2. Note that the increase in the $\beta\text{-gal}^+/\text{total nuclei}$ ratio is due to the

decrease in mononucleated cells which progressively occurs during muscle regeneration (fusion).

Table 2

Time(weeks)	No. of β -gal ⁺ nuclei/muscle (0% of injected cells)		No. of β -gal ⁺ nuclei/injection site (200 μ l ²) (0% of donor nuclei)	
	MyoD	satellite cells fibroblasts	MyoD	satellite cells
0	1.0x10 ⁶ (100)	1.0x10 ⁶ (100)		
1	2.1x10 ³ (0.2)	0.5x10 ³ (0.05)	8/242 (3.3)	2/256 (0.8)
2	1.8x10 ³ (0.18)	1.9x10 ³ (0.19)	7/127 (5.5)	6/123 (4.8)
4	1.6x10 ³ (0.16)	3.3x10 ³ (0.33)	6/99 (6.0)	8/105 (8.5)
8	1.9x10 ³ (0.19)	3.6x10 ³ (0.36)	7/56 (12.5)	12/60 (13.1)

Example 7**Muscle regeneration by genetically-modified fibroblasts expressing MyoD**

Fibroblasts isolated from human fetal skin were expanded and
5 transduced in vitro with a high-titer stock of the retroviral vector LBSN,
carrying a lacZ gene encoding a cytoplasmic form of β -galactosidase
(Salvatori, G., et al., 1993).

Transduction efficiency, estimated by the number of β -gal-staining
cells in culture, ranged between 40 and 70%, making further selection of
10 the transduced cells (e.g., G418 resistance) unnecessary. Transduced
fibroblasts were exposed to the adeno-MyoD vector at an m.o.i. of 2,000,
and then injected into the regenerating TA muscles of scid/bg mice (106
cells/muscle in a single injection).

The efficiency of myogenic conversion of transduced fibroblasts
15 was checked by allowing part of the cell culture to differentiate in vitro.
On average, about 70% of the fibroblasts underwent myogenic conversion
in these conditions, and most myotubes (> 90 %) scored positive for
cytoplasmic β -gal expression. After 1, 2 and 4 weeks, mice were
sacrificed and the TA serially sectioned and tibialis anterior muscles were
20 cut and stained for β -gal activity.

Two weeks after injection, β -gal+ fibers were observed in 7 out of
8 injected muscles. Higher magnification clearly revealed fibers
accumulating the reporter gene product at variable levels, suggesting a
variability in the proportion of injected/host cells in the β -gal+ fibers.

25 The presence and contribution of human cells in the newly formed
fibers was confirmed by immunocytochemical analysis using an antibody
which recognizes human but not mouse fetal myosin heavy chains, which

showed patches of intensive expression of human muscle-specific proteins in the regenerating areas.

In general, the average number of β -gal+ fibers per muscle obtained by injecting human converted fibroblasts was lower than that observed after injection of murine fibroblasts, although comparable to the number of positive cells observed in experiments in which lacZ-transduced human satellite cells were used. This indicates that human cells are, as expected, less efficient than murine ones in colonizing a mouse muscle.

However, and more importantly, human converted fibroblasts perform like primary myogenic cells *in vivo*, in terms of both number and size of newly formed fibers.

In order to better quantify the survival of human myogenically converted fibroblasts in mouse muscle, the Applicant also performed dot-blot Southern analysis with an Alu probe, as previously described (Salvatori, G., et al., 1993). To this purpose DNA was extracted from TA muscles of scid/bg mice two weeks after the injection of MyoD-converted human fibroblasts (following the same experimental protocol described above). As a positive control, the same number of human satellite cells was injected in similarly treated contra-lateral TA muscles.

The results of the dot-blot indicated that approximately 0.1% of the DNA extracted from TA injected with converted fibroblasts was of human origin; on the other hand this value raised to 0.3% in the case of the sample injected with human satellite cells.

Because histochemical analysis had revealed that virtually all the β -galactosidase staining was inside muscle fibers, we can conclude that the observed values faithfully reflect the percentage of human nuclei

incorporated into regenerated muscle fibers.

Example 8

Immune response against cells exposed ex vivo to the adenoviral vector.

5 One of the major problems associated to the in vivo use of adenoviral vectors in immunocompetent recipients is the induction of a significant, T-cell-mediated immune response against both the viral proteins and the product of the transgene(s). In the model described herein, no viral particle is injected directly in vivo. However, injected 10 cells had been exposed shortly before administration to the adenoviral vector in vitro.

Replication-defective adenoviral DNA is expected to be rapidly diluted, and eventually lost, in cells in active cell division. Expression of the MyoD transgene, however, blocks almost immediately cell division in 15 the converted fibroblasts, thus preventing the possibility of diluting the adenoviral vector by continuous culture.

To test whether an immune response could be raised by cells expressing a defective adenoviral genome in vivo, the Applicant injected 20 2×10^6 C3H murine skin fibroblasts converted to myogenesis by exposure to the adeno-MyoD vector in the regenerating muscle of singeneic mice.

Cells were labelled with DiI before injection and not with a lacZ transgene to avoid a possible immune reaction against the β -galactosidase protein. Animals were sacrificed 7, 14 and 21 days after the injection, and 25 muscles analysed for the presence of immune infiltrate by immunofluorescence using antibodies against cell surface markers of murine leukocytes and macrophages.

No significant immune infiltrate was detected for up to 3 weeks after injection around the labelled cells, even though by this time all treated mice had developed antibodies against Ad5 proteins, which recognise adeno-infected but not control C3H fibroblasts

5 These experiments indicate that fibroblasts converted in vitro by exposure to an adeno-MyoD vector, and administered in vivo by intramuscular injection, do not elicit, and are therefore unlikely to be eliminated by, a cell-mediated cytotoxic immune response.

In contrast, direct injection of Adenoviral vector into regenerating
10 muscle induced a strong immune infiltrate, as already reported (Yang, Y.,
et al., 1996).

BIBLIOGRAPHY

- Salvatori, G., G. Ferrari, A. Mezzogiorno, S. Serivdei, M. Coletta, P. Tonali, R. Giavazzi, G. Cossu, and F. Mavilio. 1993. Retroviral vector-mediated gene transfer into human primary myogenic cells leads to expression in muscle fibers in vivo. *Human Gene Ther.* 4:713-723.
- Webster, C. e, H.M. Blau 1990. Accelerated age-related decline in replicative life span of Duchenne muscular dystrophy myoblasts: implication for cell and gene therapy. *Somatic Cell Mol. Genet.* 16:557-565.
- 10 Davis, R. L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51:987-1000.
- Cossu, G., S. Tajbakhsh, and M. Buckingham. 1996. Myogenic specification in mammals. *Trends Genet.* 12:218-223.
- 15 Cossu, G. 1997. Unorthodox myogenesis: possible developmental significance and implications for tissue histogenesis and regeneration. *Histol. Histopathol.* 12:755-760.
- Breton, M., Z., Li, D. Paulin, J.A. Harris, F. Rieger, M. Pincon-Raymond, and L. Garcia. 1995. Myotube driven myogenic recruitment of 20 cells during in vitro myogenesis. *Develop. Dynam.* 202:126-136.
- Gibson, A.J., J. Karasinski, J. Relvas, J. Moss, T.G. Sherratt, P.N. Strong, and D.J. Watt. 1995. Dermal fibroblasts convert to a myogenic lineage in mdx mouse muscle. *J. Cell Science* 108:207-214.
- 25 Salvatori, G., L. Lattanzi, M. Coletta, S. Aguanno, E. Vivarelli, R. Kelly, G. Ferrari, J. A. Harris, F. Mavilio, M. Molinaro, and G. Cossu. 1995. Myogenic conversion of mammalian fibroblasts induced by differentiating muscle cells. *J. Cell Science* 108:2733-2739.

- Murry, C. E., M. A. Kay, T. Bartosek, S. D. Hauschka, and S. M. Schwartz. 1996. Muscle differentiation during repair of myocardial necrosis in rats via gene transfer with MyoD. *J. Clin. Invest.* 98:2209-2217.
- 5 Cusella - De Angelis, M.G., S. Molinari, A. Ledonne, M. Coletta, E. Vivarelli, M. Bouchè, M. Molinaro, S. Ferrari, and G. Cossu, 1994. Differential response of embryonic and fetal myoblasts to TGF β : a possible regulatory mechanism of skeletal muscle histogenesis. *Development* 120:925-933.
- 10 Bader, D., T. Masaki, and D. A. Fischman. 1982. Immunohistochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* 95:763-770.
- 15 Hasty, P., A. Bradley, J. H. Morris, D. G. Edmondson, J. M. Venuti, E. N. Olson, and W. H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* 375:787-790.
- Edom, F., V. Mouly, J. P. Babert, M. Y. Fiszman, and G. S. Butler-Browne. 1994. Clones of human satellite cells can express in vitro both fast and slow myosin heavy chains. *Develop. Biology* 164:219-229.
- 20 Yasin, R., K. C. Van Beers, C. E. Nurse, S. Al-Ani, D. N. Landon, and E. J. Thompson. 1977. A quantitative technique for growing human adult skeletal muscle in culture starting from mononucleated cells. *J. Neurol. Sci.* 32:347-360.
- 25 Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. *Anal. Biochem.* 162:156-159.
- Bober, E., G.E. Lyons, T. Braun, G. Cossu, M. Buckingham, and

- H.H. Arnold. 1991. The muscle regulatory gene Myf-6 has a biphasic pattern of expression during early mouse development. *J. Cell Biol.* 113:1255-1265.
- Boulter, J., W. Luyten, K. Evans, P. Mason, M. Ballivet, D.
5 Goldman, D. Stengelin, S. Martin, S. Heinemann, and J. Patric, 1985. Isolation of a clone coding for the α -subunit of a mouse acetylcholine receptor. *J. Neurosci.* 5:2545-2552.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed., Cold Spring Harbor Laboratory
10 Press, New York.
- Yang, Y., S. E. Haecker, Q. Su, and J. Wilson, 1996. Immunology of gene therapy with adenoviral vectors. *Hum. Mol. Genet.* 5:1703-1712.
- Tajbakhsh, S., E. Vivarelli, M. G. Cusella-De Angelis, D.
Rocancourt, M. Buckingham, and G. Cossu 1994. A population of
15 myogenic cells derived from the mouse neural tube. *Neuron* 13:813-821.
- Lyons, G. E., S. Muhlebach, A. Moser, R., Masood, B. M.
Paterson, M. Buckingham, and J. C. Perriard. 1991. Development regulation of creatin kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. *Development* 113:1017-
20 1029.

CLAIMS

1. A method for the preparation of genetically-modified fibroblasts expressing a muscle lineage commitment gene, which comprises:
 - 5 a) ex-vivo transduction of fibroblasts with a therapeutic gene or a gene capable of correcting a gene defect;
 - b) transient expression of the muscle lineage commitment gene in fibroblasts transduced as at point (a), through transformation of the cells with a high-efficiency DNA transfer method, wherein the muscle lineage commitment gene is under the control of a strong promoter.
- 10 2. A method according to claim 1, wherein the therapeutical gene is the dystrophin gene.
- 15 3. A method according to claim 1, wherein the high-efficiency DNA transfer method is a viral vector.
4. A method according to claim 3, wherein said viral vector is selected from baculovirus, adeno-related viruses, adeno-virus.
5. A method according to claim 3, wherein said vector is an adenovirus.
- 20 6. A method according to claim 1, wherein the muscle lineage commitment gene is selected from MyoD, Myf-5, MRF4 and myogenin.
7. A method according to claim 6, wherein said gene is MyoD.
8. A method according to claim 1, wherein the muscle lineage commitment gene is under the control of a viral promoter.
- 25 9. Genetically-modified fibroblasts transiently expressing a muscle lineage commitment gene.
10. Fibroblasts according to claim 9, wherein the muscle lineage

commitment gene is MyoD.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/03075

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/10 //C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 12979 A (UNIV SOUTHERN CALIFORNIA) 18 May 1995 (1995-05-18) cited in the application the whole document page 14, line 10 - page 15, line 4 -----	9,10
Y	WO 96 09373 A (BRITISH TECH GROUP ;WATT DIANA JOAN (GB); BETTENCOURT DE MEDEIROS) 28 March 1996 (1996-03-28) cited in the application the whole document -----	1-10
Y	WO 98 17784 A (UNIV LAVAL ;TREMBLAY JACQUES P (CA)) 30 April 1998 (1998-04-30) the whole document -----	1-10
A	-----	-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the International search

23 August 1999

Date of mailing of the International search report

13/09/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Bilang, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/03075

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 219 740 A (MILLER A DUSTY ET AL) 15 June 1993 (1993-06-15) the whole document ----	
A	WEINTRAUB H ET AL: "ACTIVATION OF MUSCLE-SPECIFIC GENES IN PIGMENT, NERVE, FAT, LIVER, AND FIBROBLAST CELL LINES BY FORCED EXPRESSION OF MYOD" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 86, 1 July 1989 (1989-07-01), pages 5434-5438, XP000672860 ISSN: 0027-8424 the whole document ----	
P,X	LATTANZI L, ET AL.: "High Efficiency Myogenic Conversion of Human Fibroblasts by Adenoviral Vector-Mediated MyoD gene transfer" JOURNAL OF CLINICAL INVESTIGATION, vol. 101, no. 10, 15 May 1998 (1998-05-15), pages 2119-2128, XP002112711 page 2124 - page 2125 page 2127, column 2, paragraph 1 ----	1-10
P,X	HUARD C, ET AL.: "Transplantation of Dermal Fibroblasts Expressing MyoD1 in Mouse Muscles" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 248, no. 3, 30 July 1998 (1998-07-30), pages 648-654, XP002112712 page 651, column 2, paragraph 1 ----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte:	inal Application No
	PCT/EP 99/03075

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 9512979	A	18-05-1995	AU	1052795 A		29-05-1995
WO 9609373	A	28-03-1996	AU	694957 B		06-08-1998
			AU	3481695 A		09-04-1996
			CA	2198379 A		28-03-1996
			EP	0783568 A		16-07-1997
			GB	2293604 A,B		03-04-1996
			JP	10505756 T		09-06-1998
WO 9817784	A	30-04-1998	AU	4613397 A		15-05-1998
US 5219740	A	15-06-1993		NONE		

THIS PAGE BLANK (USPTO)